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Association between the Expression Pattern of Two Fibroblast Growth Factor 2 (FGF_2) and Patterns Estrogen Receptor 1 (ESR_1) Promising Key Genes in Sheep with Extra Functional Teats

Research Article

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ABSTRACT

The nursing ability of productive ewes has been a subject of extensive research. From today's perspective, the survivability of multiple lambs of highly productive ewes in birth time depends largely on the number of functional teats. Different expression patterns in a range of candidate genes may act as a regulatory signal for a particular phenotype from a molecular genetic perspective. A molecular study was developed to validate the supernumerary teats characteristics. The second research phase includes a case-control application study to analyze the gene expression patterns of fibroblast growth factor 2 (FGF_2) and expression of estrogen receptor 1 (ESR_1) genes. The approach utilized standard laboratory techniques for RNA extraction, cDNA synthesis and cyber green dye-based gene expression quantification. When comparing two routine teat groups with four teats observation of the gene expression profile clearly shows the FGF_2 gene pattern ($P<0.01$), There was no obvious difference in ESR_1 gene response between the two experimental groups. This means that by combining promising gene expression tools, molecular approaches and evidence to validate gene expression in ewe carry supernumerary teats which will ultimately improve sheep production. Future research could provide valuable insights into the literature.

KEY WORDS ESR_1 gene, FGF_2 gene, sheep, teat number.

INTRODUCTION

Large litters of sheep often result in ewes being unable to afford to nurse their newborn lambs, as physiological capacity has been shown to be the main factor determining lamb viability (Ahbara *et al.* 2019). Therefore, developing sheep breeds with four functional teats could be a way to address the problem of orphaned lambs, which is a disadvantage of large litter sizes in sheep (Bakhtiarizadeh *et al.* 2020). All young animals should have access to sufficient nutrients before weaning if the litter size exceeds the number of functional teats (insufficient number of teats) (Chatterjee *et al.* 2019). However, since this was not the case, there was a very high risk of child mortality (Dysin *et al.* 2021). In addition, pre-weaning mortality increases due to competition from newborn lambs for access to narrow udders. From these data, it can be concluded that improved suckling performance and udder freedom are achieved when a mother gives birth to multiple lambs and has four healthy, functioning teats. This means that it can be very difficult for multiple offspring to be viable if there are four functional teats in ewes. In certain cases, an alternative approach must be taken, such as raising orphans (Ghaffarilaleh *et al.* 2022).

To understand the genetic basis of variation, teat variation must be analyzed at the DNA level and search for putative mutations in candidate genes. Knowledge about potential hub genes that control mammary gland differentiation and maturation from epithelial cells is still quite limited. Therefore, exploring potential genes involved in the embryonic development of the mammary gland will shed light on the process of epithelial differentiation. Fibroblast growth factor (FGF), T-box transcription factor, parathyroid hormone-related protein (PTHrP), and wingless integration site (WNT) are essential for mammary bud formation and maintenance (Eydivandi *et al.* 2022).

The FGF_2 and ESR_1 genes, which have been identified as coding genes related to the regulation of mammary gland development, embryogenesis, lactation characteristics, and ovarian follicle survival and maturation, have been introduced into research so far.

FGF is crucial for regulating teat morphogenesis, proliferation and differentiation (Peng *et al.* 2017). In particular, FGF_2, found on chromosome 17, has three exon and two intron regions. There is a significant degree of nucleotide and protein sequence similarity between the molecular structure of the FGF_2 gene and that of bovine and buffalo species (Pértille *et al.* 2017). A 155 amino acid protein is encoded by the 468 bp ORF that forms the FGF_2 coding sequence. The signal peptide consists of 10155 amino acids, 146 of which are among the first 19 that have properties. In addition, important intracellular signaling pathways such as the Ras/MAP kinase, PLC/PKA, STAT and/or PI3-kinase signaling pathways are linked to the FGF-FGFR ligand (Purcell *et al.* 2007; Smołucha *et al.* 2021).

According to a review of previous research, mammary gland development is regulated by the estrogen receptor and its receptor (Mueller *et al.* 2002). After ovariectomy, the calf's mammary gland did not develop; however, it recovered after an estrogen receptor injection, according to a review of previous research. The estrogen receptor and its receptor are regulatory factors of mammary gland development (Gagniac *et al.* 2020). A review of previous research found that the estrogen receptor and its receptor regulate mammary gland development. After an estrogen receptor injection, the calf's mammary gland, which had not developed after the oophorectomy, recovered. Ovariectomy also caused the expression of estrogen receptor 1 (ESR_1) in the mammary epithelial cells (MECs) of dairy goats (Zhang *et al.* 2019). The ESR_1 gene is essential for follicular growth and successful ovulation in ewes and is a key gene for estrogen biosynthesis. It works similarly to ESR_2 (Xu *et al.* 2018). The ESR_1 gene, which is essential for follicle growth and successful ovulation in ewes, functions similarly to ESR_2 as a key gene for estrogen biosynthesis. The ESR_1 gene was found in Chr. 18 and included 10 introns and 11 exons

The Russian Romanov sheep breed takes its name from the city of the same name. This breed of sheep, native to Northern Europe, is suitable for the cold. They have short tails. Romanov males typically weigh between 55 and 80 kg, while females typically weigh between 40 and 50 kg (Deniskova *et al.* 2017). Iran has recently seen an increase in the crossing of this breed with native breeds. However, reports have surfaced about the intersection's inconsistent results.

With this in mind, the current scenarios point us to use actual gene expression data to search for potential biomarkers associated with the number of functional sheep teats. Part of the research phase is a case-control application study, the aim of which is to analyze the FGF_2 and ESR_1 gene expression patterns.

MATERIALS AND METHODS

Animals

In order to fully convey the complexity of the phenomenon related to the Romanov sheep breed, a case study methodology was chosen. Of the 20 healthy ewes in the control group, there were two normal teats, and 15 Romanov ewes with four functional teats formed the case group. Figure 1 illustrated supernumerary teats (polythelia) in Romanov sheep with four functional teats and normal milk secretion.

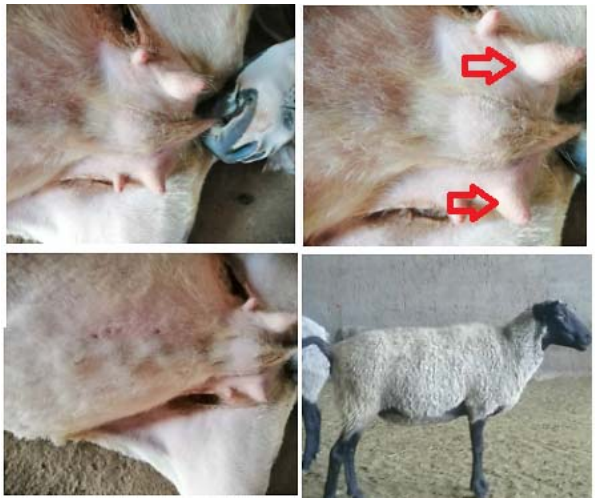

Figure 1 Romanov sheep with four teats (supernumerary teats) and normal milk secretion, the main teats are marked with arrows on the figure

Chemicals, RNA extraction and FGF_2 and ESR_1 genes expression profile

The consumables and chemicals used in this study were supplied by Merck Group and Sigma-Aldrich Company.

Table 1 illustrated an overview of candidate genes examined, sequences of primers and their associated properties. According to the manufacturer's instructions, total RNA was isolated from the cells using a commercially available RNA kit from Rocheh and the RNA was then eluted in 50 µL RNase-free water. The RNA concentration was determined using NanoDrop and the suitability of the RNA for sequencing was assessed using a bioanalyzer. Messenger RNA was converted to cDNA using the SuperScript II Reverse Transcriptase Kit. In summary, 2 µL of dt primer was mixed with 1 µg of total RNA and diluted with water to give a final volume of 10 µL. After 10 minutes at 70 °C and 8 µL master reaction mix, the sample was denatured. The Rocheh Real-Time PCR System was used to examine qRT-PCR gene expression using SYBR Green-based real-time PCR. Each sample was treated with 10 µL reaction solution containing 10 ng DNA, 5 spots, 6 µL homemade SYBR green mix, and 3 pmol/µL primer pair for each of the two candidate genes. The number of cycles required to exceed a given fluorescence signal threshold (also called Threshold Cycle C_T) is directly proportional to the amount of DNA used in this experiment. The relative n-fold change can be calculated using the Livak formula Rq= 2^{-delta} delta ct, since the number of cycles correlates exponentially with the RNA in each candidate gene in two groups of two and four functional teats. The above software applications were used to create the graphs: GraphPad PRISM 6 or "R" version 3.3. 0.

Statistical analysis

The qPCR data were analyzed using the ANOVA test and the mean differences were compared using the T test ($p<0.05$). The data for the gene expression experiment of each group were generated using SAS Ver. generated. 9.2 software (SAS, 2003). $P<0.05$ was considered a significant value.

RESULTS AND DISCUSSION

Two candidate genes (*EFG2* and *ESR1*) and the housekeeping gene b-actin showed the expected PCR size, without artificial or nonspecific bands, during the gene expression experiment in a 2.5% agarose gel.

During the gene expression experiment, the expected PCR size for two candidate genes (EFG_2 and ESR_1) and the b-actin housekeeping gene was observed in 2.5% agarose gel with no artificial or unspecific bands. Figure 2 illustrates qPCR gene expression results for FGF_2 expression in sheep supernumerary teats. According to Figure 2, it can be concluded that the expression of the housekeeping gene b-actin and ESR_1 gene in the two teats situation was much higher than the expression of the FGF_2 gene, but in the four teats situation, the expression of the FGF_2 gene was much higher than the other two genes.

Figure 3 shows the result of efficiency curve and R^2 for real-time PCR during running samples to measure gene expression profile. The R^2 value of 0.998 illustrated a reasonable observed input for experimental materials. Figure 4 shows observed Real time PCR curve patterns for FGF_2, ESR_1 and the b-actin genes (housekeeping gene) and behavior of ct within each case-control group.

Figure 5 addressed the comparison of ESR1 and FGF2 genes between different two and four teat groups. Observation of the gene expression profile clearly emphasizes the FGF_2 gene pattern in two routine teat groups compared to four teats ($P<0.01$).

However, the ESR_1 gene showed no significant response between the two experimental groups.

The mothering ability of highly fertile sheep breeds can be studied to learn more about the survival rate of triple and twin lameness. Few studies have thoroughly examined the role of gene expression patterns in different sheep breeds, although the effects of numerous factors affecting mortality of newborn lambs during the pre-weaning period have been thoroughly investigated. In this study, we examined the pattern and variability of expression profiles of candidate genes in Romanov ewes with two or four working teats.

The results of the study revealed interesting new information about the relationships between teat characteristics and potential gene expression patterns. When comparing two routine teat groups with four teats, observation of the gene expression profile clearly shows the FGF_2 gene pattern ($P<0.01$). However, the ESR_1 gene showed no noticeable changes between the two experimental groups. Here we compare the results of the proposed approach with those of previous related research.

Table 1 An overview of candidate genes examined, sequences of primers and their associated properties

Gene	Primer sequence (5-3)	Size bp	Annealing	Accession number	Reference
FGF2	GGGGTTGTGTCTATCAAAGGAG GTGCCACATACCAACTGGAGTA	172	60 °C, 30 s	NM_001009769.1	Mor *et al.* (2017)
ERα	GACCGAAGAGGAGGGAGAATG CGGGCTGTTCTTCTTAGTGTGTT	147	60 °C, 30 s	AY059388.1	Duan *et al.* (2019)
β-actin	AATTCCATCATGGAAGTGACG GATCTTGATCTTCATCGTGCTG	150	60 °C, 30 s	U39357.1	Mor *et al.* (2017)

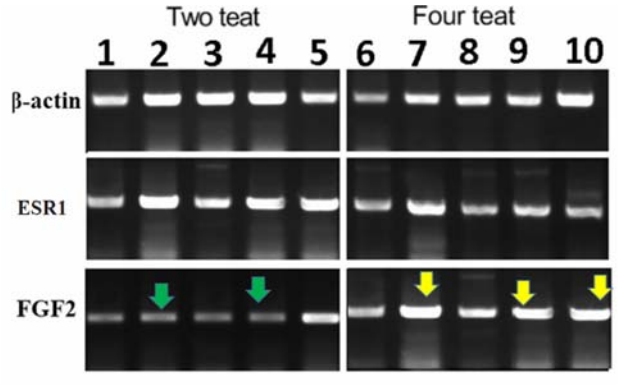

Figure 2 qPCR gene expression results for FGF-2 expression in sheep supernumerary teats

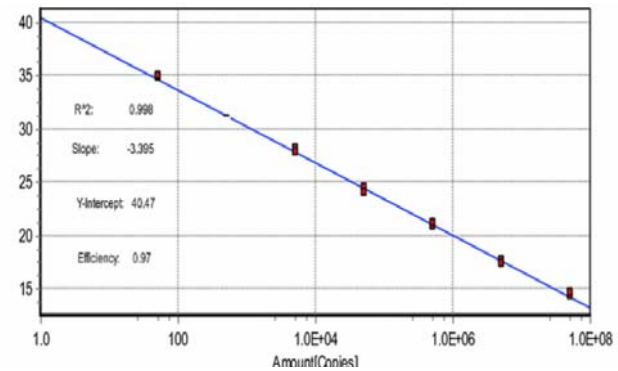

Figure 3 Outputs of general efficiency curve and R^2 for real-time PCR during running samples to measure gene expression profile

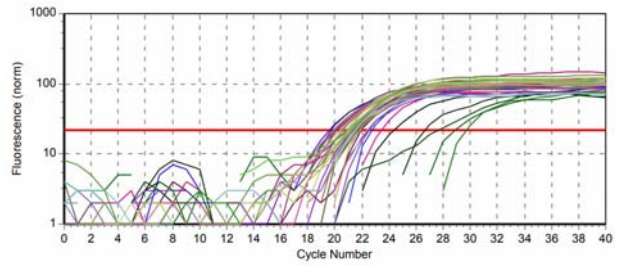

Figure 4 Real-time PCR curve patterns for EFG_2, ESR_1 and the b-actin genes (housekeeping gene) and behavior of c_t within each case-control group. B catin gene express constant Ct value trend between case and control because it was representative of housekeeping gene. ESR_1 gene expression show similar Ct value in both case and control samples and however eventually we found c_t value for EFG_2 gene in two teat ewes was significantly lower than in case group

Two steroidal sex hormones secreted by the ovaries are estrogen and testosterone. Estrogen is crucial for embryonic development and the development of secondary sexual characteristics. Receptors in target cells are necessary for regulating the physiological effects of steroid hormones.

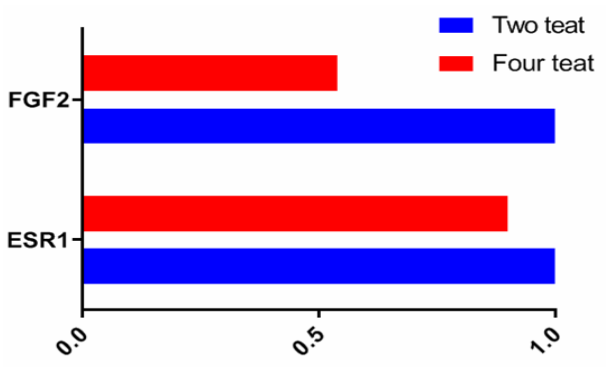

Figure 5 addressed the statistically comparison of ESR_1 and FGF_2 genes between different two and four teat groups

As a transcription factor that represents the interface between estrogen hormone secretion in many physiological processes, estrogen receptor gene expression is highly expressed in the endometrium and mammary gland (Gao *et al.* 2021). It is noteworthy that over the last decade researchers have become increasingly interested in the genetic background of supernumerary teats. We need to examine DNA and look for possible mutations in candidate genes to understand the genetic basis of SNT. Numerous studies are examining various potential genes associated with extra teats. Breast bud formation and maintenance have previously been linked to fibroblast growth factor (FGF), T-box transcription factor, parathyroid hormone-related protein (PTHrP), and wingless-related integration site (WNT) (Kenny, 2014).

Previous studies have reported that the number of teats is increased due to the potent effect of an insertion mutation in Vertebrae Development Associated (VRTN) gene on Sus scrofa chromosome 7 (SSC7) in Landrace and Korean pigs (Lee *et al.* 2014), Duroc pigs (Arakawa *et al.* 2015), Erhualian pigs (Wang *et al.* 2017) and Large White pigs (Duijvesteijn *et al.* 2014). Different genes, *SPRED2*, *MKX*, *TMSB4X* and ESR_1 are involved in this trait in Chinese Sushan pigs (Zhou *et al.* 2019). Some interesting genes related to teat number have been identified in cattle. For instance, the inheritance of supernumerary teats in Holstein cattle depends on a QTL on chromosome 20 and a polygenic portion (Jörg *et al.* 2014). Another study found that the *LGR5* gene on chromosome 5 was a candidate for the presence of supernumerary teats (Butty *et al.* 2017). In contrast to numerous studies in pigs and cattle, one study showed that BBX and CD47 on chromosome 1 were frequently identified as significant by a genome-wide association study (GWAS) in Wadi sheep (Peng *et al.* 2017). However, genetic variants associated with the number of teats in Hu sheep are not known.

Zhang *et al*. (2019) studied the expression of ESR_1, PRLR, GHR and IGF_1R in mammary glands of four-teat Hu sheep. All four receptors were mainly expressed in mammary epithelial cells and adipose cells. Furthermore, expression levels of PRLR and GHR were significantly higher in four-teat sheep during pregnancy and lactation than in two-teat sheep. Zhang data suggests that sheep with four teats ewes have more developed mammary gland tissue compared to sheep with two teats. As a logical justification, the reason for a difference between the present result and other findings seems to be due in particular to different sheep and even more to technical methods for gene expression, which can influence the analysis results. To our knowledge, this is the first report of ESR_1 and FGF_2 gene expression analysis of mammary glands from ewes naturally carrying two and four functional teats.

The small sample size of the current studies is of course one of their limitations, particularly when it comes to sheep, which amazingly have four functional teats due to the low frequency phenomena of this event. Furthermore, real-time PCR applications today pale in comparison to more advanced tools such as RNA-Seq and microarray. Laboratory and financial limitations hindered our research. Further research is required to confirm the conclusions that can be drawn from this study.

CONCLUSION

The nursing ability of productive ewes has long been the topics of research. From today's perspective, the survivability of multiple lambs of highly productive ewes in birth time depends largely on the number of functional teats. It is known that research to identify the genomic region that controls these economic traits is required to achieve high maintainability. This leads us to believe that the current scenarios based on actual gene expression data could be biomarkers for the number of functional sheep teats. We conclude that the gene expression profile in two routine teat groups as opposed to four teats clearly highlights the FGF_2 gene pattern ($P< 0.01$). There was no obvious difference in ESR_1 gene response between the two experimental groups. The mechanism regulating teat number in Romanova sheep was discovered using gene expression tools, which is a novel report to our knowledge. Further research is needed to confirm this initial discovery.

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REFERENCES

Ahbara A., Bahbahani H., Almathen F., Al Abri M., Agoub M.O., Abeba A., Kebede A., Musa H.H., Mastrangelo S., Pilla F. and Ciani E. (2019). Genome-wide variation., candidate regions and genes associated with fat deposition and tail morphology in Ethiopian indigenous sheep. *Front. Genet*. **9,** 1-8.

Arakawa A., Okumura N., Taniguchi M., Hayashi T., Hirose K., Fukawa K., Ito T., Matsumoto T., Uenishi H. and Mikawa S. (2015). Genome□wide association QTL mapping for teat number in a purebred population of Duroc pigs. *Anim. Genet*. **46(5),** 571-575.

Bakhtiarizadeh M.R., Mirzaei S., Norouzi M., Sheybani N. and Vafaei Sadi M.S. (2020). Identification of gene modules and hub genes involved in mastitis development using a systems biology approach. *Front. Genet*. **11,** 1-7.

Butty A.M., Frischknecht M., Gredler B., Neuenschwander S., Moll J., Bieber A., Baes C.F. and Seefried F.R. (2017). Genetic and genomic analysis of hyperthelia in Brown Swiss cattle. *J. Dairy Sci*. **100(1),** 402-411.

Chatterjee S.J., Halaoui R., Deagle R.C., Rejon C. and McCaffrey L. (2019). Numb regulates cell tension required for mammary duct elongation. *Biol. Open*. **8(5),** 1-9.

Deniskova T., Dotsev A.V., Selionova M., Wimmers K., Reyer H., Kharzinova V.R., Brem G. and Zinovieva N.A. (2017). 696 Whole-genome single nucleotide polymorphism study of Romanov sheep. *J. Anim. Sci*. **95(4),** 339-404.

Duan H., Xiao L., Hu J., Zhang Y., Zhao X., Ge W., Jiang Y., Song L., Yang S. and Luo W. (2019). Expression of oestrogen receptor., androgen receptor and progesterone nuclear receptor in sheep uterus during the oestrous cycle. *Reprod. Domest. Anim*. **54(10),** 1305-1312.

Duijvesteijn N., Veltmaat J.M., Knol E.F. and Harlizius B. (2014). High-resolution association mapping of number of teats in pigs reveals regions controlling vertebral development. *BMC. Genomics*. **15(1),** 1-2.

Dysin A.P., Barkova O.Y. and Pozovnikova M.V. (2021). The role of microRNAs in the mammary gland development., health., and function of cattle., goats., and sheep. *Non-Coding. RNA*. **7(4),** 78-85.

Eydivandi S., Eghbalsaied S., Momen M., Khajouei E., Javanmard A. and Ghoreishifar S.M. (2022). Search for association between ovine wingless-type MMTV integration site family member (Wnt10A) genes with supernumerary teat in Ghezel and Romanov sheep. *Iranian J. Appl. Anim. Sci*. **12(2),** 295-302.

Gagniac L., Rusidzé M., Boudou F., Cagnet S., Adlanmerini M., Jeannot P., Gaide N., Giton F., Besson A., Weyl A. and Gourdy P. (2020). Membrane expression of the estrogen receptor ERα is required for intercellular communications in the mammary epithelium. *Development.* **147(5),** 182303-182311.

Gao X., Yao X., Li X., Liang Y., Liu Z., Wang Z., Li K., Li Y., Zhang G. and Wang F. (2021). Roles of WNT6 in sheep endometrial epithelial cell cycle progression and uterine glands organogenesis. *Vet. Sci*. **8(12),** 316-321.

Ghaffarilaleh V., Javanmard A., Saberivand A., Asadzadeh N.,

Masoudi R., Barfourooshi H.J., Rashidi A. and Eghbalsaied S. (2022). Variation and frequency of supernumerary teats., litter size., histological features and the fibroblast growth factor 2 (FGF-2) gene expression pattern in goats. *Theriogenology*. **179,** 141-148.

Jörg H., Meili C., Ruprecht O., Bangerter E., Burren A. and Bigler A. (2014). A genome-wide association study reveals a QTL influencing caudal supernumerary teats in Holstein cattle. *Anim. Genet*. **45(6),** 871-873.

Kenny S. (2014). Investigation of prevalence of supernumerary teat in livestock. PhD. Thesis. University of Veterinary Medicine Budapest., Budapest, Hungary.

Lee J.B., Jung E.J., Park H.B., Jin S., Seo D.W., Ko M.S., Cho I.C., Lee J.H. and Lim H.T. (2014). Genome-wide association analysis to identify SNP markers affecting teat numbers in an F 2 intercross population between Landrace and Korean native pigs. *Mol. Biol. Rep*. **41,** 7167-7173.

Mor A., Mondal S., Reddy I.J., Nandi S. and Gupta P.S. (2018). Molecular cloning and expression of *FGF2* gene in pre-implantation developmental stages of *in vitro*-produced sheep embryos. *Reprod. Domest. Anim*. **53(4),** 895-903.

Mueller S.O., Clark J.A., Myers P.H. and Korach K.S. (2002). Mammary gland development in adult mice requires epithelial and stromal estrogen receptor α. *Endocrinology*. **143(6),** 2357-2365.

Peng W.F., Xu S.S., Ren X., Lv F.H., Xie X.L., Zhao Y.X., Zhang M., Shen Z.Q., Ren Y.L., Gao L. and Shen M. (2017). A genome-wide association study reveals candidate genes for the supernumerary nipple phenotype in sheep (*Ovis aries*). *Anim. Genet*. **48(5),** 570-579.

Pértille F., Moreira G.C., Zanella R., Nunes J.D., Boschiero C., Rovadoscki G.A., Mourão G.B., Ledur M.C. and Coutinho L.L. (2017). Genome-wide association study for performance traits in chickens using genotype by sequencing approach. *Sci. Rep*. **7(1),** 41748-41756.

Purcell S., Neale B., Todd-Brown K., Thomas L., Ferreira M.A., Bender D., Maller J., Sklar P., De Bakker P.I., Daly M.J. and Sham P.C. (2007). PLINK, a tool set for whole-genome association and population-based linkage analyses. *American. J. Hum. Genet*. **81(3),** 559-575.

SAS Institute. (2003). SAS®/STAT Software, Release 9.1. SAS Institute, Inc., Cary, NC. USA.

Smołucha G., Gurgul A., Jasielczuk I., Kawęcka A. and Miksza-Cybulska A. (2021). A genome-wide association study for prolificacy in three Polish sheep breeds. *J. Appl. Genet*. **62,** 323-326.

Wang L., Zhang Y., Zhang T., Zhang L., Yan H., Liu X. and Wang L. (2017). Genotyping by sequencing reveals a new locus for pig teat number. *Anim. Genet*. **48(4),** 470-472.

Xu S.S., Gao L., Xie X.L., Ren Y.L., Shen Z.Q., Wang F., Shen M., Eyþórsdóttir E., Hallsson J.H., Kiseleva T. and Kantanen J. (2018). Genome-wide association analyses highlight the potential for different genetic mechanisms for litter size among sheep breeds. *Front. Genet*. **9,** 118-125.

Zhang L., Peng F., Yu F., Wan L. and Zhou Z.Q. (2019). Expression of ESR1., PRLR., GHR., and IGF1R in mammary glands of Hu sheep with four teats. *Czech J. Anim. Sci*. **64(2),** 49-58.

Zhou L., Zhao W., Fu Y., Fang X., Ren S. and Ren J. (2019). Genome-wide detection of genetic loci and candidate genes for teat number and body conformation traits at birth in Chinese Sushan pigs. *Anim. Genet*. **50(6),** 753-756.